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Plastome analysis unveils Inverted Repeat (IR) expansion and positive selection in Sea Lavenders (*Limonium*, Plumbaginaceae, Limonioideae, Limonieae)

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Abstract

The genus *Limonium*, commonly known as Sea Lavenders, is one of the most species-rich genera of the family Plumbaginaceae. In this study, two new plastomes for the genus *Limonium*, viz. *L. tetragonum* and *L. bicolor*, were sequenced and compared to available *Limonium* plastomes, viz. *L. aureum* and *L. tenellum*, to understand the gene content and structural variations within the family. The loss of the *rpl16* intron and pseudogenisation of *rpl23* was observed. This study reports, for the first time, expansion of the IRs to include the *ycf1* gene in *Limonium* plastomes, incongruent with previous studies. Two positively selected genes, viz. *ndhF* and *ycf2*, were identified. Furthermore, putative barcodes are proposed for the genus, based on the nucleotide diversity of four *Limonium* plastomes.

Keywords

Intron loss, IR expansion, positive selection, pseudogenisation, *ycf1*

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Introduction

The family Plumbaginaceae of the order Caryophyllales is highly diverse, rich in species and displays a cosmopolitan distribution with its maximum diversity in the temperate areas of the northern hemisphere (Kubitzki 1993). It is sister to Polygonaceae (Lledó et al. 1998; Chase et al. 2016) and further classified into two subfamilies: Limonioideae (formerly Staticoideae) and Plumbaginoideae (Lledó et al. 1998, 2001; Hernández-Ledesma et al. 2015). Limonioideae is further divided into two tribes, Limonieae (consisting of 24 genera) and the monotypic Aegialitideae, whereas Plumbaginoideae consists of four genera. Limonioideae is a sub-cosmopolitan group distributed mostly in the Mediterranean and Indo-Turanian regions, but a few genera have also diversified in the Southern Hemisphere. *Limonium* Mill., *Acantholimon* Boiss. and *Armeria* (DC.) Willd., all belonging to Limonioideae, are the most species-rich genera, comprising 80–90% of the species in Plumbaginaceae (Koutroumpa et al. 2018).

The genus *Limonium* Mill., popularly known as sea lavenders, belongs to the subfamily Limonioideae and tribe Limonieae (Kubitzki 1993; Lledó et al. 2005; Malekmohammadi et al. 2017). The genus is represented by ca. 600 species and is the sole genus of Plumbaginaceae exhibiting a sub-cosmopolitan distribution (Koutroumpa et al. 2018). The genus comprises several ornamental and medicinally-important species (Malekmohammadi et al. 2017). *Limonium* are herbs or shrubs growing in saline or metal-rich soils, mostly in coastal areas (Kubitzki 1993). Variation of reproductive systems (sexual and apomixis), as well as events like hybridisation and polyploidy, complicate the delimitation of most of the species of *Limonium* (Kubitzki 1993; Cowan et al. 1998; Palacios et al. 2000; Akhani et al. 2013; Róis et al. 2016).

Certain phylogenetic studies tried to resolve the relationships within *Limonium* at a global scale (Lledó et al. 2005); however, many others were confined to either a specific geographic area or some specific sections of *Limonium* (Palacios et al. 2000; Lledó et al. 2005; Lledó et al. 2011; Akhani et al. 2013; Róis et al. 2016). More recently, Malekmohammadi et al. (2017) tried to resolve the phylogenetic relationships within *Limonium* including 76 species of the Mediterranean region. They found two well-supported clades for subgenera *Limonium* and *Pteroclados* (Boiss.) Pignatti, which confirmed the earlier findings by Lledó et al. (2005, 2011). Their study was based on one nuclear and several plastid loci, but lacked comprehensive sampling. Koutroumpa et al. (2018) carried out a phylogenetic study of Plumbaginaceae which included 23 genera of the family with an emphasis on the genus *Limonium* (201 spp.), based on three chloroplast and one nuclear marker. The study again confirmed most of the previous molecular phylogenies and led to the proposal of new sections and altering some of the existing sections. However, taxonomic difficulties, diversity in reproductive modes and sub-cosmopolitan distribution of *Limonium* necessitate further studies on the genus.

With the advent of sequencing technologies, the availability of large genome-scale data has made it easier to understand phylogeny and detect polyploidy events (Gompert and Mock 2017; Thomas et al. 2017; McKain et al. 2018). Five plastomes have been sequenced for Plumbaginaceae thus far, viz. *Limonium sinense* (Girard) Kuntze (Li et

al. 2020), *L. aureum* (L.) Chaz. (Zhang et al. 2020), *L. tenellum* (Turcz.) Kuntze (Yao et al. 2019) and one each of *Ceratostigma* Bunge and *Plumbago* L. The recent phylogenomic study to understand the evolution of Caryophyllales incorporated plastome sequences of *L. tenellum*, *Plumbago auriculata* Lam. and *Ceratostigma willmottianum* Stapf (Yao et al. 2019). The study reported that all Plumbaginaceae members, except *Limonium*, exhibit expanded IR to accommodate *ycf1*. However, no studies have been carried out to understand and compare the structure, composition and evolution of the plastome within Plumbaginaceae and *Limonium* in particular.

The present study reports the plastome sequences of two Asian *Limonium* species, viz. *L. tetragonum* (Thunb.) Bullock and *L. bicolor* (Bunge) Kuntze and compares the structure, composition and diversity within the genus by combining them with other available plastomes. *L. tetragonum* is a biennial species characterised by a spicate inflorescence, yellow corolla, acute calyx with pink at the base, white in upper parts and distributed in Japan, Korea, New Caledonia and Primorye (Ohwi 1965; POWO 2021). *Limonium bicolor* is a perennial species, characterised by a paniculate inflorescence, yellow corolla, somewhat rounded calyx, pink to purplish at base, white in upper parts and distributed in China and Mongolia (Wu and Raven 1996; POWO 2021). The former is known for its anti-cancerous properties (Kong et al. 2008; Bae et al. 2016), while the latter is most studied for its salt glands (Li et al. 2019; Lu et al. 2020). An attempt has also been made to unravel the structural variations within Plumbaginaceae plastomes and propose molecular markers for easier discrimination of *Limonium* species.

Material and methods

Sampling and sequencing

Leaf samples of *Limonium bicolor* and *L. tetragonum* were collected from Meneng steppe of Dornod Province of Mongolia (Voucher No. KRIB 0070251) in June 2015 and from the coastal area of Ulsan City of the Republic of Korea (Voucher No. KRIB 0086343) in April 2018, respectively. The samples were deposited at the Herbarium of Korea Research Institute of Bioscience and Biotechnology (KRIB). DNA extraction was carried out from dried leaves using the DNeasy Plant Mini Kit (QIAGEN, Cat. No. 69104) according to the manufacturer's protocol. For both the plastomes, a 550 bp DNA TruSeq Illumina (Illumina, San Diego, CA, USA) sequencing library was constructed. After the library preparation, the DNA samples were run in a single lane of an Illumina HiSeq 10X with a read length of 151 bp.

Assembly and annotation

The raw reads obtained after Illumina sequencing were analysed using FastQC V0.11.7 (Andrews 2010) software to ensure the quality of the reads and Phred score. The assembly was carried out using NOVOPlasty V4.2 (Dierckxsens et al. 2017). Forward

and reverse reads with a read length of 150 bp and insert size of 300 bp, as well as seed sequence (*rbcL* of *L. tetragonum*), were used as input. *De novo*, as well as reference-based assembly, were carried out. The plastome sequence of *L. aureum* (MN623109) was used as a reference for the assembly of both the plastomes. The assembly and orientation of the Inverted Repeats (IRs), Large Single Copy (LSC) and Small Single Copy (SSC) regions were confirmed by NCBI blast and graphic view using Geneious prime 2020.2.2 (https://www.geneious.com). The assembled plastomes were annotated using Geneious prime 2020.2.2 (https://www.geneious.com) and Geseq – Annotation of Organellar Genomes (Tillich et al. 2017). The transfer RNAs (tRNAs) were identified with tRNA-scan-SE (Lowe and Chan 2016). Graphical maps of both plastomes were visualised using OGDRAW (Lohse et al. 2013).

Comparative plastome analysis

All Plumbaginaceae plastomes available so far were included for comparison. Four plastomes of *Limonium*, viz. *L. aureum* (MN623109), *L. tenellum* (MK397871), *L. tetragonum*, *L. bicolor* and *Ceratostigma willmottianum* (MK397862), as well as *Plumbago auriculata* (MH286308), were included in the analysis. The plastome of *L. sinense* was not included in any of the analyses due to ambiguities observed in the assembly. The selected six plastomes were aligned using a Geneious prime 2020.2.2 plugin MAFFT v.7.450 (Katoh and Standley 2013). The contraction and expansion of IRs at the junction sites were examined and plotted using IRscope (Amiryousefi et al. 2018). Percentage sequence identity for all six plastomes was plotted using MultiPIPMaker (http://pipmaker.bx.psu.edu/pipmaker/) considering *L. tetragonum* as a reference (Schwartz et al. 2003).

Identification of Simple Sequence Repeats (SSRs) and repeats

Simple Sequence Repeats across the four *Limonium* plastomes were detected using MISA online server (Beier et al. 2017). The minimum repeat units for mono-, di-, tri-, tetra-, penta- and hexanucleotide repeats were set as 10, 5, 4, 3, 3 and 3, respectively, whereas forward, reverse, palindromic and complementary repeats were identified using the programme REPuter (Kurtz et al. 2001). The threshold for repeat length was set to 30 with more than 90% similarity and the hamming distance was set to 3.

Nucleotide diversity

The four *Limonium* plastomes were aligned using Geneious prime 2020.2.2 plugin MAFFT v.7.450 (Katoh and Standley 2013). The aligned sequences were curated manually and, due to the presence of ambiguous *ycf1* region in *L. tenellum* at IRb/SSC junction, the sequences were trimmed for all four taxa before the analysis. Nucleotide diversity and sliding window analysis were performed using the software DnaSP v.6.12.01 (Rozas et al. 2017).

Codon usage

The percentage codon usage of protein-coding regions of all four *Limonium* plastomes was calculated using Geneious prime 2020.2.2. To examine the frequency and uniformity of Synonymous codon and codon biases, the Relative Synonymous Codon Usage (RSCU) was also determined in DnaSP v.6.12.01 software (Rozas et al. 2017).

Positive selection analysis

In order to detect protein-coding genes under selection in the genus *Limonium*, sequences of each gene were aligned using the MAFFT v.7.450 plugin of Geneious prime 2020.2.2. The aligned sequences were again manually checked for an end-to-end alignment. The phylogenetic tree for each protein-coding gene was constructed using the FastTree plugin (Price et al. 2010) of Geneious prime 2020.2.2. The site-specific model was performed using CODEML algorithms (Yang 1998) implemented in EasyCodeML software (Gao et al. 2019). Seven codon substitution models (M0, M1a, M2a, M3, M7, M8 and M8a) were investigated and compared to identify positively selected sites, based on likelihood ratio tests.

Phylogenomic analysis

A total of 39 plastome sequences, including six from Plumbaginaceae were considered as ingroups for phylogenomic analysis. The outgroup was composed of members of Amaranthaceae. All the plastome sequences were aligned using the MAFFT v.7.450 plugin of Geneious prime 2020.2.2. Maximum Likelihood analysis, based on the best fit model GTR+F+R5, was performed using IQtree 1.6.12-MacOSX (Nguyen et al. 2015). The best likelihood score for the consensus tree was -979751.011.

Results

General features of the plastomes

The average organelle coverage for the plastomes of *L. tetragonum* and *L. bicolor* was 1014X and 1009X, respectively. Plastomes of *L. tetragonum* and *L. bicolor* exhibited a typical quadripartite structure (Fig. 1). Total plastome lengths of *L. tetragonum* and *L. bicolor* were 154,691 bp and 154,617 bp with LSC (84,568 bp and 84,541 bp), SSC (12,997 bp and 12,964 bp) and a pair of IRs (28,563 bp and 28,556 bp), respectively (Table 1). The GC content of both plastomes was 37%. Both the plastomes exhibited 83 protein coding genes, 37 tRNA genes and four rRNA genes (duplicated in IR region) (Tables 1, 2). The assembled and annotated plastomes of *L. tetragonum* and *L. bicolor* were deposited to the NCBI database with accession numbers MW085088 and MW085089, respectively.

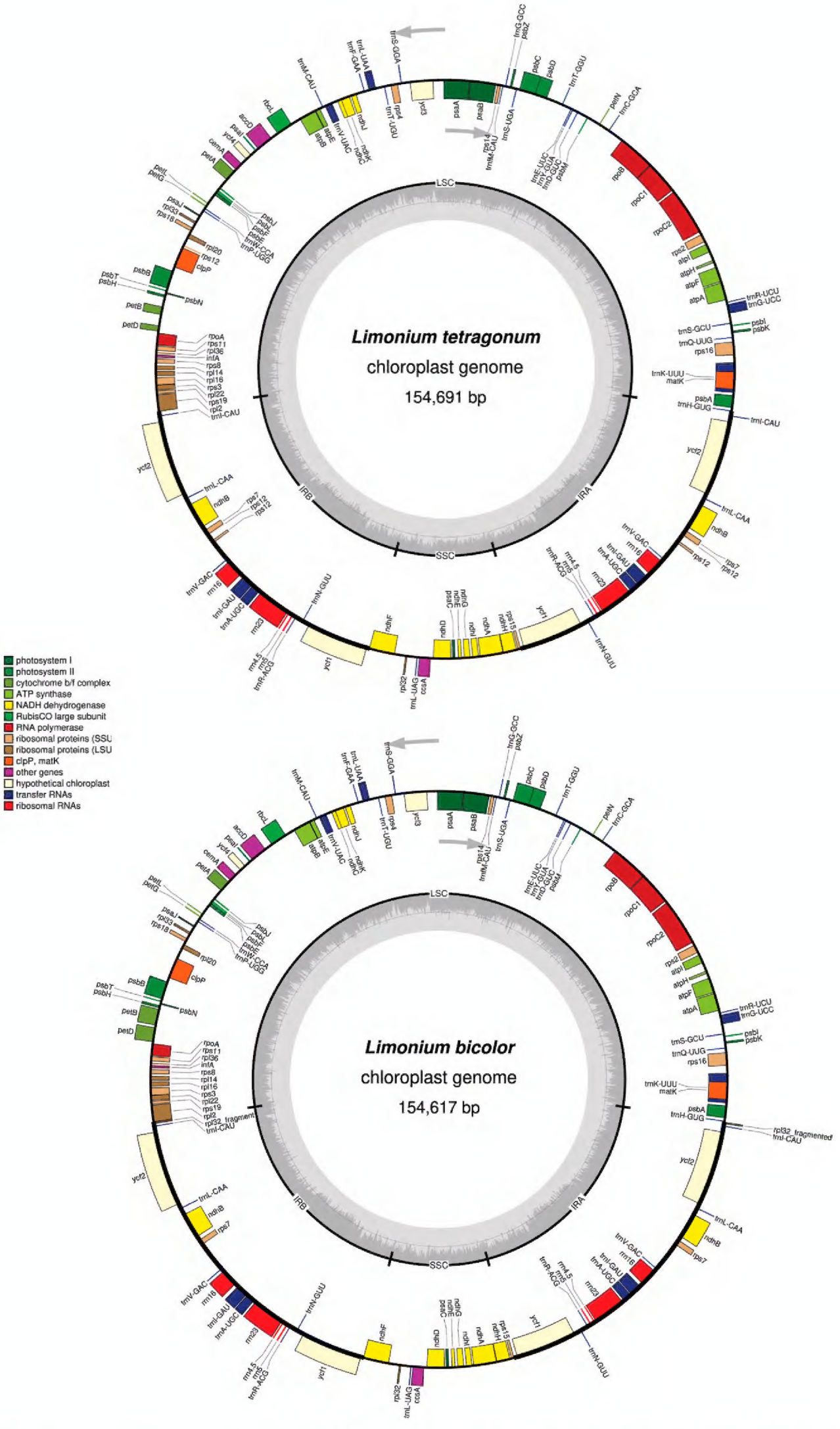

Figure 1. Circular gene map of plastomes of *L. tetragonum* and *L. bicolor*. Genes drawn inside the circle are transcribed clockwise and those outside are counter-clockwise. Genes belonging to different functional groups are shown in different colours. The innermost circle denotes GC content across the plastome.

Table 1. Comparison of plastome features of Plumbaginaceae members.

Species	*Limonium tetragonum*	*Limonium bicolor*	*Limonium aureum*	*Limonium tenellum*	*Ceratostigma willmottianum*	*Plumbago auriculata*
Accession No.	MW085088	MW085089	MN623109	MK397871	NC041261	NC041245
Genome size (bp)	154691	154617	154661	150515	164999	168765
LSC length (bp)	84568	84541	84546	84634	89454	91912
SSC length (bp)	12997	12964	12980	23755	13491	13331
IR length (bp)	28563	28556	28568	21063	31027	31761
No. of genes duplicated in IR	15	15	16	10	15	19
No. of genes	128	128	130	124	127	132
No. of protein coding genes	83	83	83	82	82	84
No. of tRNA genes	37	37	37	36	37	37
No. of rRNA genes	8	8	8	6*	8	8
Total GC content (%)	37	37	37.1	37.1	37.5	37.2

*indicates annotations available as per NCBI database.

Table 2. List of genes in the newly-sequenced plastomes of *L. tetragonum* and *L. bicolor*.

Category	Group	Name
Photosynthesis-related genes	Rubisco	*rbcL*
	Photosystem 1	*psaA*, *psaB*, *psaC*, *psaI*, *psaJ*
	Photosystem 2	*psbA*, *psbB*, *psbC*, *psbD*, *psbE*, *psbF*, *psbH*, *psbI*, *psbJ*, *psbK*, *psbL*, *psbM*, *psbN*, *psbT*, *psbZ*
	APT synthase	*atpA*, *atpB*, *atpE*, *atpF*†, *atpH*, *atpI*
	Cytochrome b/f complex	*petA*, *petB*, *petD*, *petG*, *petL*, *petN*
	NADPH Dehydrogenase	*ndhA*†, *ndhB*†*, *ndhC*, *ndhD*, *ndhE*, *ndhF*, *ndhG*, *ndhH*, *ndhI*, *ndhJ*, *ndhK*
Transcription and translation-related genes	Transcription	*rpoA*, *rpoB*, *rpoC1*†, *rpoC2*
	Ribosomal proteins	*rps2*, *prs3*, *rps4*, *rps7**, *rps8*, *rps11*, *rps12*†*, *rps14*, *rps15*, *rps16*†, *rps18*, *rps19*
		rpl2†, *rpl14*, *rpl16*, *rpl20*, *rpl22*, *rpl23#**, *rpl33*, *rpl36*
	Translation initiation factor	*infA*
RNA genes	Ribosomal RNA	*rrn5**, *rrn4.5**, *rrn16**, *rrn23**
	Transfer RNA	*trnA-UGC*†*, *trnC-GCA*, *trnD-GUC*, *trnE-UUC*, *trnF-GAA*, *trnfM-CAU*, *trnG-UCC*, *trnH-GUG*, *trnI-GAU*†*, *trnK-UUU*†, *trnL-CAA**, *trnL-UAA*, *trnL-UAG*†, *trnM-CAU*, *trnN-GUU**, *trnP-UGG*, *trnQ-UUG*, *trnR-ACG**, *trnR-UCU*, *trnS-GCU*, *trnS-GGA*, *trnS-UGA*, *trnT-GGU*, *trnT-UGU*, *trnV-GAC**, *trnW-CCA*, *trnY-GUA*
Other genes	RNA processing	*matK*
	Carbon metabolism	*cemA*
	Fatty acid synthesis	*accD*
	Proteolysis	*clpP*†
Genes of unknown function	Conserved reading frame	*ycf1*†*, *ycf2**, *ycf3*†, *ycf4*

*duplicated genes, †genes with introns, #pseudogenised genes.

IR expansion and contraction

The IR regions of plastomes are divided by four junctions viz., IRb/LSC, IRb/SSC, IRa/SSC and IRa/LSC. All six plastomes of Plumbaginaceae (including four *Limonium*) were compared for their IR boundaries. The annotations available on NCBI database were used for *Limonium aureum*, *L. tenellum*, *Ceratostigma willmottianum* and *Plumbago auriculata*.

The IRb/LSC junction of three *Limonium* species viz. *L. tetragonum*, *L. bicolor* and *L. aureum* was characterised by the presence of the *rpl2* gene (Fig. 2). The gene also extends in IRb with 11 bp, 10 bp and 11 bp, respectively. However, in *L. tenellum* and

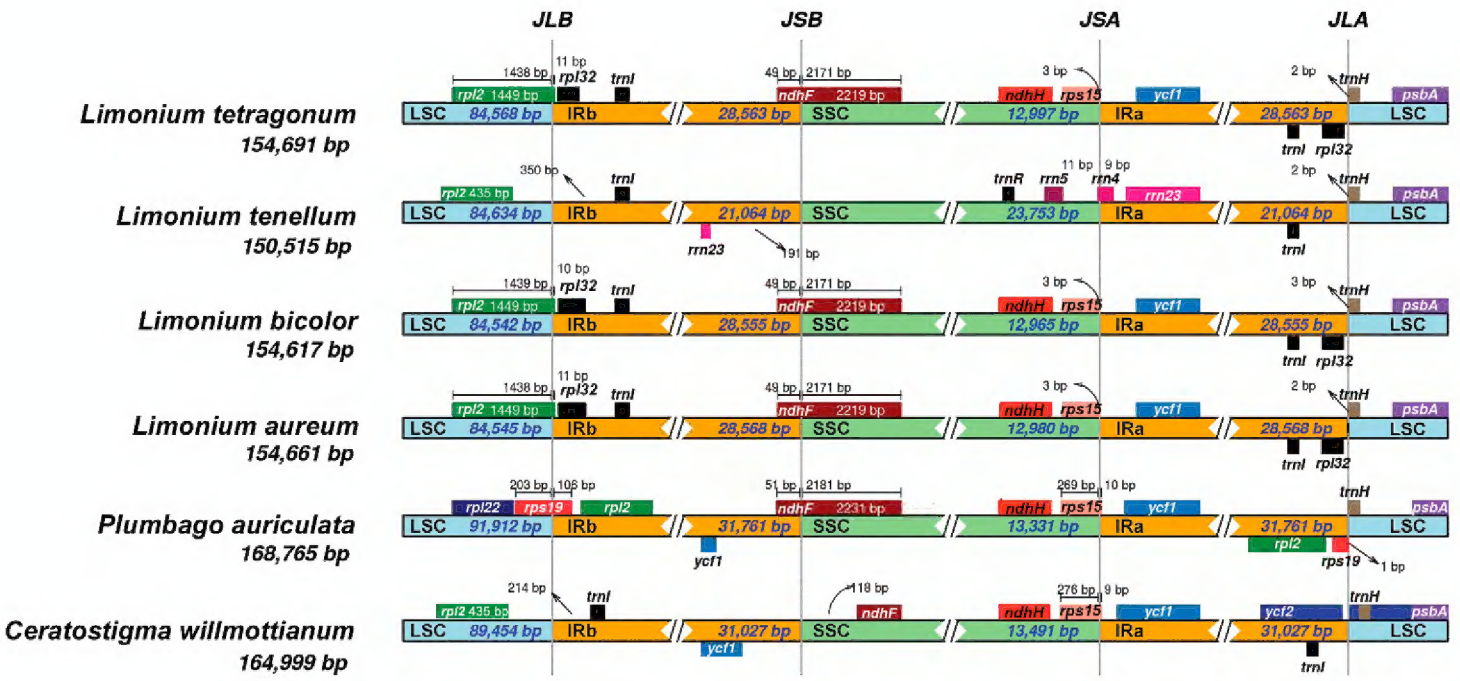

Figure 2. Comparison of IRs of Plumbaginaceae plastomes.

C. willmottianum, it was exclusively present in the LSC region, 937 bp and 1,041 bp away from the junction, respectively. In *Plumbago*, IR was expanded to include the *rps19* gene at the junction, while *rpl2* was duplicated in the IR region.

The next junction, i.e. IRb/SSC, was characteried by the presence of the gene in *L. tetragonum*, *L. bicolor*, *L. aureum* and *P. auriculata* (49 bp of all *Limonium* species and 51 bp of *Plumbago* in IR region). In *L. tenellum*, the junction exhibited *rrn23* (IR) and *trnR-ACG* (SSC), which could probably be due to wrong assembly or annotation. In *Ceratostigma*, *ndhF* appeared to be shifted to SSC, 118 bp away from IRb border.

IRa/SSC junction of all compared species was characterised by the presence of *rps15* and *ycf1* genes, except in *L. tenellum*. The IRa/LSC junction was characterised by *rpl32* and *trnH* in *L. tetragonum*, *L. bicolor* and *L. aureum*, while in *L. tenellum* and *C. willmottianum*, it was characterised by *ycf2* and *trnH*. In *P. auriculata*, the junction was bordered by *rps19* and *trnH* (Fig. 2).

Plastomes of *L. aureum*, *L. bicolor* and *L. tetragonum* exhibited two copies of *ycf1*, except for *L. tenellum* which exhibited a single copy. All three plastomes are characterised by the *ycf1* gene having a length of 5,298 bp and IR has been expanded to accommodate the *ycf1* gene. *Plumbago* and *Ceratostigma* also exhibited the *ycf1* gene duplicated in IRs. However, the annotation provided for *L. tenellum* (MK397871) exhibits a single copy of *ycf1*.

Structural comparison

Plastomes of *Limonium tetragonum*, *L. tenellum*, *L. bicolor* and *L. aureum* were compared with two Plumbaginaceae plastomes, keeping *L. tetragonum* as a reference. Sequence divergence amongst the four compared *Limonium* plastomes was similar as compared to *Plumbago* and *Ceratostigma*. *Limonium tenellum* exhibited partial deletion at the IRa/LSC junction in the *ycf1* gene (Fig. 3). Our results suggest that the gene *rpl23* has been pseudogenised in all the studied plastomes of the three genera of Plumbaginaceae. The length of *rpl23* in all four *Limonium* plastomes was observed to be 171 bp, 270 bp in *Plumbago* and 50 bp in *Ceratostigma*. A similar loss of intron was observed in *L. aureum*,

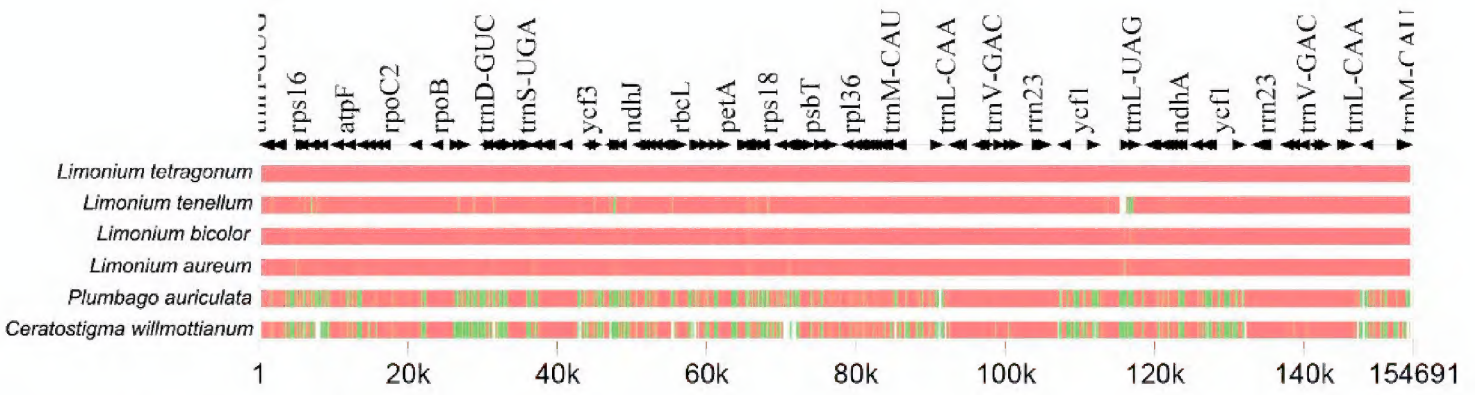

Figure 3. Plastome alignment of Plumbaginaceae members.

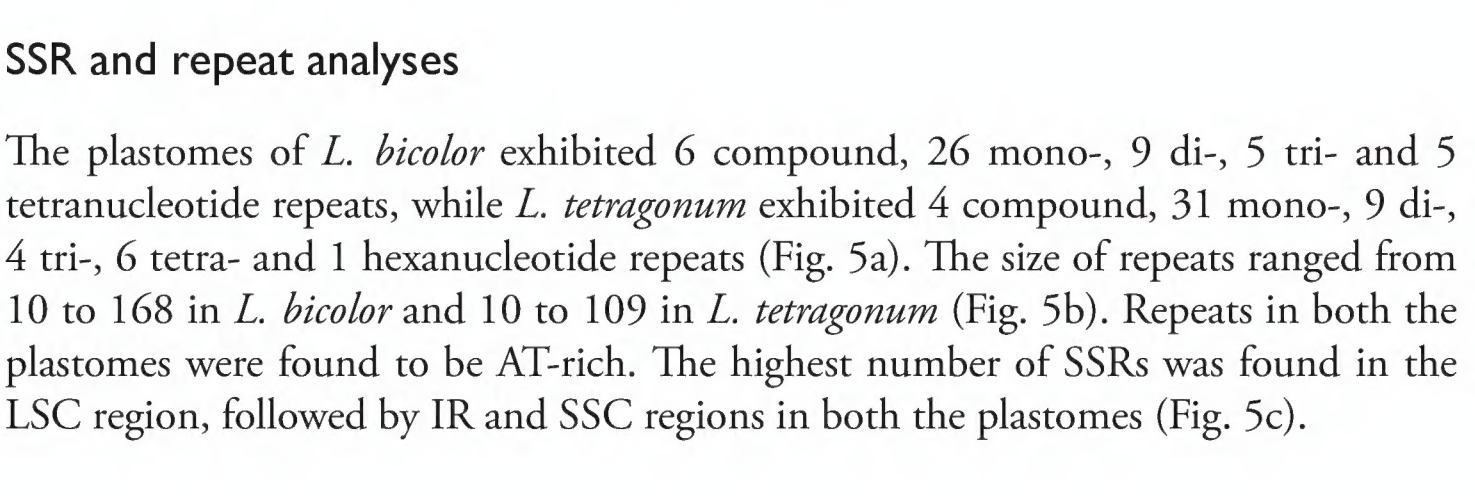

Figure 4. Nucleotide diversity and hotspot regions between *Limonium* plastomes. The X-axis represents the nucleotide position and Y-axis represents nucleotide diversity (Pi).

L. bicolor and *L. tetragonum* in our study. In all four compared species of *Limonium*, the length of *rpl16* was 408 bp without any intron. However, the two other genera of Plumbaginaceae, i.e. *Plumbago* and *Ceratostigma*, exhibited the presence of intron. The length of the gene was 1,449 bp in the former, while in the latter, it was 1,469 bp.

Nucleotide diversity

The nucleotide diversity (Pi) of four *Limonium* plastomes was analysed, except for the *ycf1* region, which was removed due to ambiguous alignment. Sliding window analysis yielded some regions with higher Pi values. High nucleotide diversity was found in two spacer regions viz. *trnY-GUA-trnT-GGU*, *trnL-UAG-ccsA* and one gene *ycf3* with Pi values 0.015, 0.043 and 0.02, respectively (Fig. 4).

SSR and repeat analyses

The plastomes of *L. bicolor* exhibited 6 compound, 26 mono-, 9 di-, 5 tri- and 5 tetranucleotide repeats, while *L. tetragonum* exhibited 4 compound, 31 mono-, 9 di-, 4 tri-, 6 tetra- and 1 hexanucleotide repeats (Fig. 5a). The size of repeats ranged from 10 to 168 in *L. bicolor* and 10 to 109 in *L. tetragonum* (Fig. 5b). Repeats in both the plastomes were found to be AT-rich. The highest number of SSRs was found in the LSC region, followed by IR and SSC regions in both the plastomes (Fig. 5c).

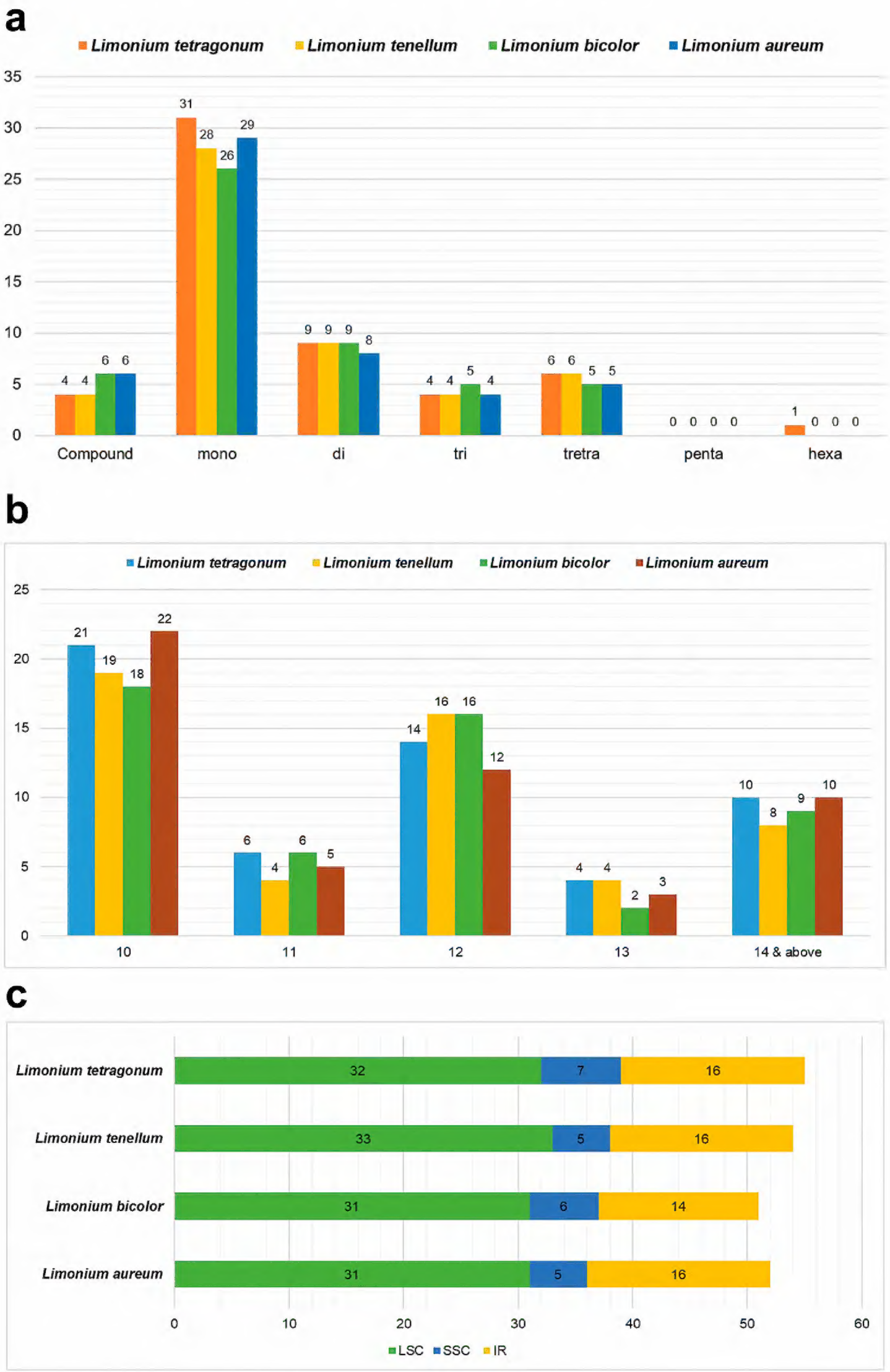

Figure 5. Repeat analysis of four *Limonium* plastomes. a. Types of repeats, b. Size of repeats, c. Position of repeats.

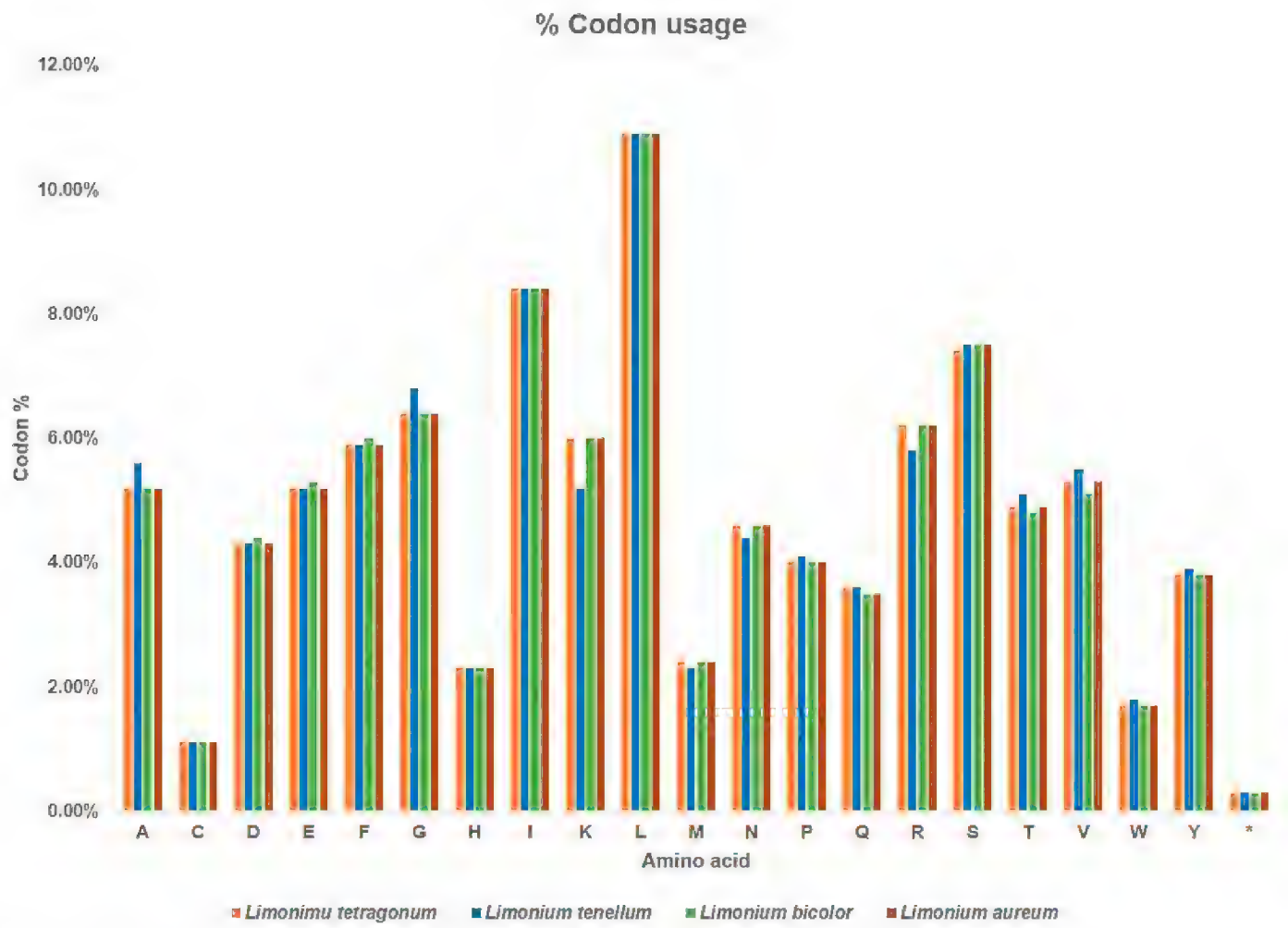

Figure 6. Codon usage of four *Limonium* species. X-axis: Amino acid, Y-axis: codon usage in percentage. * indicates the stop codon.

All four species of *Limonium* exhibited only forward and palindrome type in RE-Puter analysis (Suppl. material 1).

Codon usage

The four *Limonium* plastomes were compared for their codon usage. The plastomes of *L. tetragonum*, *L. tenellum*, *L. bicolor* and *L. aureum* exhibited 27,290, 24,093, 26,682 and 27,308 codons, respectively. Leucine was the most abundant while Cysteine was the least abundant amino acid in all the compared plastomes (Fig. 6). Codon usage was biased towards A and T in all the compared plastomes. The highest codon preference was 1.88, 1.89, 1.82 and 1.91 while the lowest was 0.33, 0.36, 0.37 and 0.36, respectively, in *L. tetragonum*, *L. tenellum*, *L. bicolor* and *L. aureum* (Suppl. material 2). Codon usage was biased towards A and T in all the compared plastomes.

Positive selection analysis

A total of 79 consensus protein-coding genes of four *Limonium* species were evaluated with respect to selective pressure. Two genes were found to have undergone positive selection viz. *ndhF* and *ycf2* with ω values 2.2278 and 19.657, respectively (Suppl. material 3).

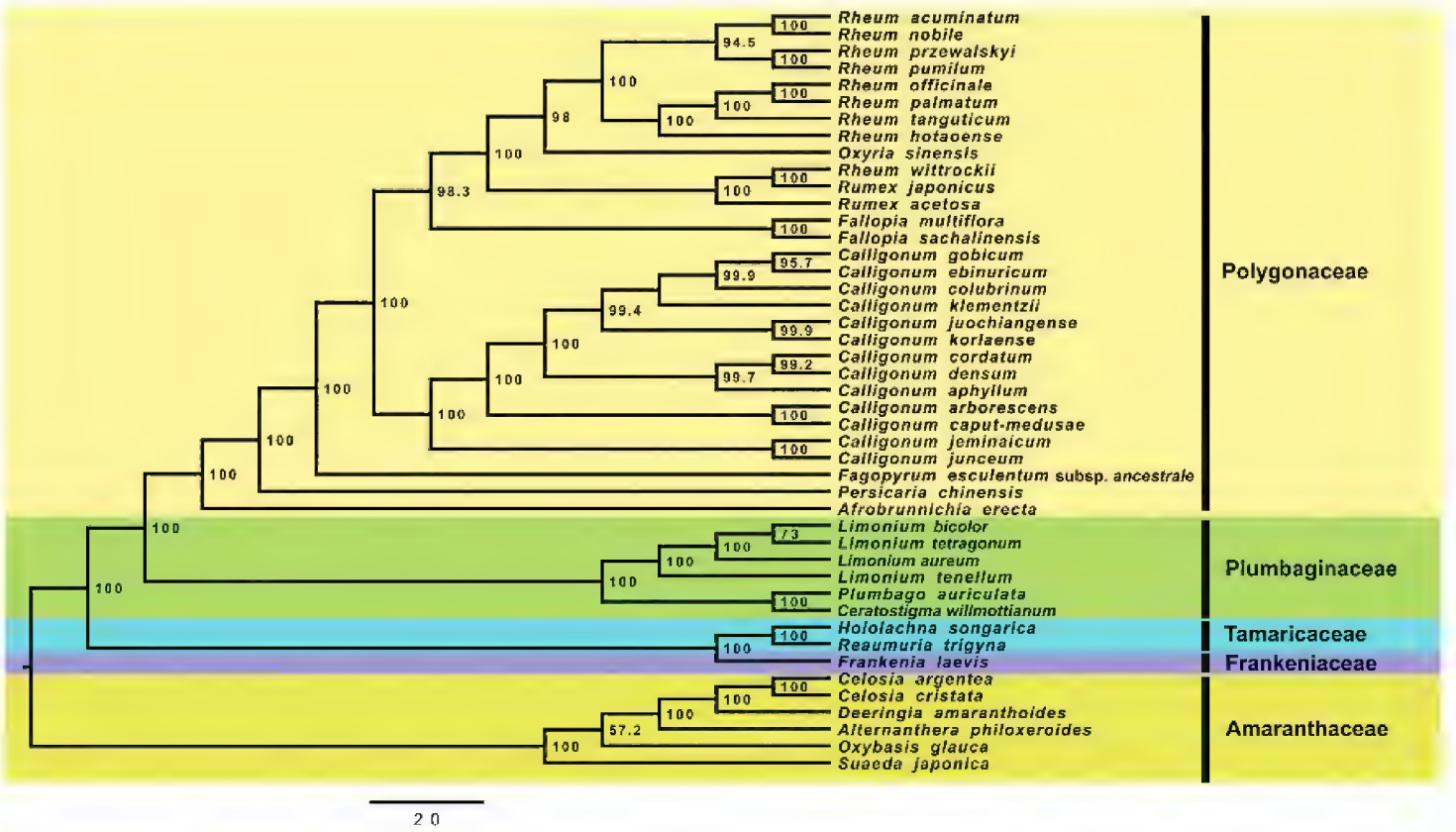

Figure 7. Phylogenomic tree from the Maximum Likelihood analysis showing the placement of the studied four *Limonium* species. Nodal support is represented by bootstrap percentages.

Phylogenomic analysis

In the phylogenomic analysis, the representatives of *Limonium* formed a strongly-supported monophyletic group (BS = 100), in which *L. bicolor* was recovered as sister to *L. tetragonum* (BS = 73), with *L. aureum* and *L. tenellum* being successive sisters (BS = 100) to the clade of *L. bicolor* and *L. tetragonum*. *Plumbago auriculata* and *Ceratostigma willmottianum* also formed a monophyletic group (BS = 100), sister to the *Limonium* clade (BS = 100). All these made Plumbaginaceae a strongly-supported (BS = 100) monophyletic group (Fig. 7).

Discussion

In this study, two *Limonium* plastomes were assembled and the structure and composition of four *Limonium* plastomes were compared. The plastomes were conserved in terms of size and structure ranging from 154,617 to 154,691 bp, except for *L. tenellum* with 150,515 bp. Expansion and contraction of IRs/SSC account for huge variation, evolutionary events and also affect the plastome sizes (Zhu et al. 2016; Darshetkar et al. 2019; Maurya et al. 2020). Yao et al. (2019) reported the expansion of IRs in Polygonaceae and Plumbaginaceae members to accommodate *ycf1*, except for the genus *Limonium*. However, the study included a single plastome sequence of *Limonium tenellum*. In all the *Limonium* plastomes, except *L. tenellum*, IR has been expanded to accommodate *ycf1*, unlike that reported by Yao et al. (2019). We assume the single copy of *ycf1* in *L. tenellum* could be a result of erroneous assembly and annotation. Sequencing more plastomes for the genus will help to better understand the arrangement and position of *ycf1* in *Limonium*.

Ribosomal Protein L23 is a protein component of the 60s large ribosomal subunit. The comprehensive study of plastomes of Caryophyllales (Yao et al. 2019) reported that the gene has undergone pseudogenisation at least 11 times in the order. Pseudogenisation has also been reported in the family Polygonaceae, a sister family of Plumbaginaceae (Logacheva et al. 2008). The results of our study corroborate these earlier studies. Transfer of the functional copy of the gene to the nucleus has been reported for many plastid genes in angiosperms (Millen et al. 2001; Jansen et al. 2011; Daniell et al. 2016). The functional copy of *rpl23* might have been transferred to the nucleus, even in the members of Plumbaginaceae as predicted by Yao et al. (2019). Ribosomal Protein L16 codes for a protein component of the 50s ribosomal subunit. The loss of *rpl16* intron was reported in *Limonium gmelinii* Kuntze and *Limonium latifolium* Kuntze (Campagna and Downie 1998). Recently, Yao et al. (2019) reported the loss of *rpl16* intron in *L. tenellum* plastome. Hence, our study confirms the loss of *rpl16* intron on the branch leading to *Limonium* as reported by Yao et al. (2019).

The value of the ratio of synonymous and nonsynonymous substitutions (Ka/Ks or ω) above 1 indicates that the corresponding genes experience positive selection, however, ω values ranging from 0.5 to 1 indicate relaxed selection (Tomoko 1995). The *ndhF* gene was found to be positively selected in the genus *Rheum* L. of Polygonaceae. The study reported that the higher expression levels of *ndhF* were observed in *Rheum* under environmental stress conditions (Li et al. 2016). Most of the *Limonium* species also grow in saline soils, which could be the reason behind the adaptive evolution of the gene. Positive selection of the *ycf2* gene has already been reported in several angiosperms (Jiang et al. 2018; Zhong et al. 2019).

The sampling for the phylogenomic analysis followed the studies of Walker et al. (2018) and Yao et al. (2019). The ingroup consisted of plastomes belonging to the FTPP clade, i.e. Frankeniaceae, Tamaricaceae, Plumbaginaceae and Polygonaceae (they were all recovered as strongly-supported monophyletic groups), while the outgroup consisted of species belonging to Amaranthaceae. The position of the four *Limonium* species studied fits with the earlier studies (Yao et al. 2019). In the present study, phylogenetic position of four *Limonium* species belonging to subgenus *Limonium* (Koutroumpa et al. 2018) was studied. A study based on *rbcL*, *trnL* intron and *trnL-F* intergenic spacer included two species viz. *L. tenellum* and *L. tetragonum*, which were resolved in the same clade, but were placed into two different subsections, according to Boissier (1848), *Rhodanthae* and *Chrysanthae*, respectively. However, the study treated them under subgenus *Limonium* (Lledo et al. 2005). Later, Malekmohammadi et al. (2017) studied the phylogeny for the genus, based on several plastid and single nuclear (ITS) markers. The study included *L. tetragonum* and *L. aureum* and both the species were placed in the "*L. aureum* clade". Recently, Koutroumpa et al. (2018) carried out phylogenetic studies of the genus, based on three chloroplast (*trnL-F*, *matK* and *rbcL*) and one nuclear marker (ITS). Three species, *L. tetragonum*, *L. aureum* and *L. tenellum* were included in the study. These three species were all resolved in the same clade. These species were earlier placed under sect. *Plathymenium*, *L. tetragonum* and *L. aureum* belonging to subsect. *Chrysanthae* and *L. tenellum* under subsect. *Rhodanthae* (Boissier 1848). Earlier studies reported plastome data dealing with single *Limonium* species

and placed them as sister to *Plumbago* species (Yao et al. 2019; Li et al. 2020; Zhang et al. 2020). The present phylogenetic study, for the first time, included *L. bicolor* and analysed four *Limonium* plastomes together. However, inclusion of more *Limonium* plastomes would help in understanding the intrageneric relationships.

Conclusions

The present study has made an effort to understand the structural changes in the plastomes of Plumbaginaceae by including two newly-generated *Limonium* plastome sequences. The study also confirms the loss of *rpl16* intron in the genus *Limonium* and pseudogenisation of the *rpl23* gene in Plumbaginaceae. Our results also revealed, for the first time, the expansion of the IRs to accommodate the *ycf1* gene in *Limonium* as in other Plumbaginaceae members. The annotation available for *L. tenellum* exhibits *ycf1* in the SSC region. Hence, the sequencing of more plastomes would aid in identifying the exact position of *ycf1*. Two positively-selected genes were identified, viz. *ndhF* and *ycf2*. The positive selection of these genes could be linked to the evolution of *ndhF* to adapt to extreme environmental conditions, such as salt stress. It would be interesting to identify the adaptive sites in the *ndhF* amino acid by adding more *ndhF* sequences of *Limonium* species, while the expansion of IRs and accommodation of *ycf2* genes could be related to the re-arrangement of the plastome. The function of *ycf2* is still not clear, but it would be interesting to study the *ycf2* evolution and re-arrangement in the whole order. High nucleotide diversity was observed in two spacer regions *trnY-GUA--trnT-GGU*, *trnL-UAG--ccsA* and one gene *ycf3*, which could be used as potential DNA barcodes for the genus. Future studies will focus on identifying adaptive codon sites in positively-selected genes and correlating those with the habitats and environmental conditions and validation of the proposed barcodes by including more *Limonium* species.

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Supplementary material 1

The output of the repeat analysis of four *Limonium* plastomes

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Data type: molecular analysis

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Link: https://doi.org/10.3897/phytokeys.175.61054.suppl1

Supplementary material 2

Relative Synonymous Codon Usage

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Data type: molecular data

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Supplementary material 3

List of Positively selected genes

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Data type: molecular data

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